Reference: *Biol. Bull.* **198:** 67–76. (February 2000)

The Apical Sensory Organ of a Gastropod Veliger Is a Receptor for Settlement Cues

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Abstract. On the basis of anatomy and larval behavior, the apical sensory organ (ASO) of gastropod veliger larvae has been implicated as the site of perception of cues for settlement and metamorphosis. Until now, there have been no experimental data to support this hypothesis. In this study, cells in the ASO of veliger larvae of the tropical nudibranch *Phestilla sibogae* were stained with the styryl vital dye DASPEI and then irradiated with a narrow excitatory light beam on a fluorescence microscope. When its ASO cells were bleached by irradiation for 20 min or longer, an otherwise healthy larva was no longer able to respond to the usual metamorphic cue, a soluble metabolite from a coral prey of the adult nudibranch. The irradiated cells absorbed the dye acridine orange, suggesting that they were dying. When larvae not stained with DASPEI were similarly irradiated, or when stained larvae were irradiated with the light beam focused on other parts of the body, there was no loss of ability to metamorphose. Together these data provide strong support for the hypothesis. Potassium and cesium ions, known to induce metamorphosis in larvae of many marine-invertebrate phyla, continue to induce metamorphosis in larvae that have lost the ability to respond to the coral inducer due to staining and irradiation. These results demonstrate that (1) the ASO-ablated larvae have not lost the ability to metamorphose and (2) the ions do not act only on the metamorphic-signal receptor cells, but at other sites downstream in the metamorphic signal transduction pathway.

Introduction

Abundant data demonstrate that most invertebrate larvae succeed in locating appropriate habitats for settlement, metamorphosis, and growth by responding to site-specific chemical cues associated with conspecific individuals, requisite prey, microbial films, or algal or other benthic substrata (Crisp, 1974, 1984; Pawlik, 1992; Hadfield, 1998). Much current research on the subject of larval settlement focuses on the chemical identity of settlement cues and the signal-transduction mechanisms by which external cues stimulate morphogenetic transformations in the larva (*e.g.*, see papers in *Biofouling* 12(1), 1998). Despite more than 50 years of research on metamorphic induction in marine-invertebrate larvae, experimental definition of the exact location on the larval body where these interactions take place—that is, where the chemoreception that results in site-specific larval settlement occurs—is lacking for most groups. The anterior pole of at least some cnidarian planulae includes a region that must contact stimulatory surfaces for metamorphosis to occur (Müller *et al.*, 1977; Freeman and Ridgway, 1987), and there is good evidence that the antennules of barnacle cyprid larvae are the location of receptors for settlement inducers (summarized by Clare, 1995). However, the site of induction has eluded strong inference for the trochophores and trochophore-derived larvae of large marine-invertebrate clades.

Larvae of many phyla behave prior to settlement in a manner that indicates that they are "testing" or "sampling" substrata. For larvae of groups as diverse as phoronids, polychaetes, and chitons, all of which possess an apical tuft of elongate and somewhat stiff cilia, presettlement behavior typically includes swimming near the substratum with their apical ends downward so that the apical tuft brushes or is pressed against the substratum (Barnes and Gonor, 1973; Nott, 1973; Zimmer, 1991; unpublished personal observations on the serpulid polychaete *Hydroides elegans*). Thus the apical tuft, together with its underlying cells, has long been suspected to be the site of detection of substrate-associated cues for settlement. As others have, we refer to

Received 1 July 1999; accepted 3 November 1999.

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the apical tuft and its associated cells as the apical sensory organ, henceforth abbreviated ASO.

Larvae of opisthobranch gastropods bear a probable sensory structure, derived developmentally and evolutionarily from the ASO of trochophore larvae (Bonar, 1978). Recent papers by Kempf *et al.* (1997) and Marois and Carew (1997) provide elegant details concerning the cellular composition of the ASOs of opisthobranch larvae. This ASO of opisthobranch larvae consists of a set of cilia-bearing receptor cells, some of which send axons directly into the cerebral commissure of the brain; neurons from other ASO cells innervate the velum. Bonar (1978) noted how well situated the structure is, in veliger larvae of the nudibranch *Phestilla sibogae* Bergh 1905, to detect the water-borne chemical cue that arises from the nudibranch's coral prey and induces metamorphosis. An ASO has been demonstrated in prosobranch larvae as well (Uthe, 1995), where Leise (1996) reported that it can be stained with DASPEI, a styryl fluorescent dye.

The only previous experimental data supporting the hypothesis that the ASO is the site of cell-surface receptors for inducers of metamorphosis in invertebrate larvae were provided by Baxter and Morse (1992) for larvae of the gastropod *Haliotis rufescens*. Their experiments revealed that receptors for lysine, a compound known to modify the effects of a metamorphic inducer for which GABA is a receptor agonist, lie on cilia harvested from the larvae of *H. rufescens;* among these cilia were those from the apical tuft. Larvae of the nudibranch *Onchidoris bilamellata* were reported to detect a water-borne cue from the barnacle prey of the adult nudibranch *via* a pair of lateral propodial "ganglia"; however, the barnacle factor induces only reversible settlement behavior, but not metamorphosis, which is dependent on a surface-bound cue (Arkett *et al.*, 1989).

Because all authors who have described details of ASOs in gastropod larvae have argued for a probable role for this organ in induction of settlement and metamorphosis, it is timely to perform robust experimental tests of the hypothesis. In the research presented here, the vital dye DASPEI, known to vitally stain mitochondria (Haugland, 1996) and thus mitochondria-rich sensory cells (Bereiter-Hahn, 1976; Nurse and Faraway, 1989; Balak *et al.*, 1990; Leise, 1996; Boudko *et al.*, 1999), was employed to vitally stain the ASO in veliger larvae of *Phestilla sibogae*, followed by photoablation of the stained organ by fluorescent excitation. Balak *et al.* (1990) reported that labeling lateral-line hair cells of amphibians with DASPEI and exposing them to epifluorscent illumination at 450–490 nm resulted in cell death due to phototoxic conditions arising from photoexcitation of DASPEI.

We attempted to obtain additional evidence of cell death in the ASO by applying the vital dye acridine orange to treated larvae. Acridine orange, long used as a fluorochrome indicator of cell death, becomes intercalated into uncoiled DNA of dying cells when applied at relatively low (*i.e., ca.* 10^{-6} *M*) concentrations (Delic *et al.*, 1991). Although acridine orange can stain RNA, as well as DNA, the red-orange (~650 nm) emission color of the RNA–acridine-orange complex distinguishes it from DNA–acridine-orange emission, which is green (525 nm) (Haugland, 1996). Although some experimentalists have reported acridine-orange staining to be specific to cells undergoing apoptosis (*e.g.*, Abrams *et al.*, 1993), others report similarity of staining between different forms of induced cell death, at least in later stages (reviewed by Darzynkiewicz and Traganos, 1998).

Among the major tools used to study metamorphosis in marine invertebrates has been a growing list of so-called artificial inducers (reviewed by Crisp, 1974, 1984; Pawlik, 1992; Hadfield, 1998). The most useful of these are the cations potassium and cesium, which induce metamorphosis in larvae from seven phyla in the absence of other stimuli (summarized by Herrmann, 1995; Woollacott and Hadfield, 1996). While noting that the entire larval bodies are bathed in elevated potassium or cesium when larvae are treated with these ions, some authors have proposed that K^+ or Cs^+ act by depolarizing the sensory cells that typically bind the natural inducer and thus initiate spikes in neurons extending from those cells (*e.g.*, Baloun and Morse, 1984; Yool *et al.*, 1986; Leitz and Klingmann, 1990; Herrmann, 1995; Woollacott and Hadfield, 1996; Carpizo-Ituarte and Hadfield, 1998). The possibility that K^+ or Cs^+ acts on the entire nervous system (Todd *et al.*, 1991), directly on target tissues (Yool *et al.*, 1986), or at intermediate sites downstream from primary receptors (Jensen *et al.*, 1990; Pechenik *et al.*, 1995) has also been noted. Experimental evidence delineating the site of potassium activity has been difficult to obtain. In the research reported here, we exposed larvae whose ASOs had been photoablated to elevated potassium levels or to cesium in seawater to determine if the larvae could still be induced to metamorphose by these ions.

Phestilla sibogae is an appropriate model organism for studies of the sensory pathway involved in metamorphic induction, because its developmental biology, with special emphasis on induction and activation of metamorphosis, is so well known (*e.g.*, Hadfield, 1978; Hadfield and Pennington, 1990; Miller and Hadfield, 1990; Pires and Hadfield, 1993). In the research reported here, we provide the first experimental evidence that the ASO is the site of reception of external cues to settlement and metamorphosis in larvae of *P. sibogae*, and, by inference, probably in many other larvae that bear prominent apical tufts of cilia.

Materials and Methods

Larval culture. Populations of the tropical Indo-Pacific nudibranch *Phestilla sibogae* are continuously maintained in the authors' laboratory at the Kewalo Marine Laboratory,

Honolulu, Hawaii. Adult animals are kept in shallow trays supplied with constantly flowing, unfiltered seawater and with their coral prey, *Porites compressa* Dana 1846, which is collected from the field biweekly. These animals reproduce continuously, each adult laying one to two egg masses per day, and each egg mass containing 2000 to 4000 fertilized eggs. Larvae develop to normal hatching in 6 to 8 days, varying with ambient temperature (annual range = 23°–27°C). After hatching, larvae are maintained in aerated filtered seawater (FSW) containing the antibiotics streptomycin (500 mg/l) and penicillin (50 mg/l) until they have developed the capacity to undergo metamorphosis (*i.e.*, are competent) when appropriately stimulated, typically about 9 days postfertilization (Miller and Hadfield, 1986). In the experiments described here, larvae were used in experiments when 10 or 11 days old.

Labeling ASO cells with DASPEI. Ten- or eleven-day-old larvae of *P. sibogae* were placed in artificial seawater with pH reduced to 5.6 for 1.5 h during which their larval shells were decalcified, a process that does not otherwise injure the larvae, and then returned to natural seawater (Pennington and Hadfield, 1989; Pires and Hadfield, 1993). Because shell-less larvae cannot retract, they cannot conceal the ASO from the irradiation treatment described below. Cells in the ASO were labeled by bathing up to 200 larvae in 50 ml of 0.5 m*M* DASPEI (absorption maximum 461 nm; Molecular Probes Inc., Eugene, OR) in FSW for 30 min, and then rinsing them in 100 ml of FSW for one hour.

Immobilization of larvae. Labeled larvae were immobilized by embedding them in a thin (2–4 mm) layer of 1% low-melting-point agarose (Type VII, Sigma Chemical Co.) in FSW across the bottom of a 10 × 35 mm plastic petri dish (Falcon). The agarose gels at 25°–28°C, which is within the normal range of sea temperature in Hawaii. Survival of larvae in this treatment was usually 100%. To protect the agarose gel from drying, 2 ml of FSW was layered on top of it in each petri dish.

Irradiation of labeled cells. A Zeiss inverted microscope equipped with a mercury light source and fluorescence filters was used to visualize the larvae, selectively irradiate DASPEI-labeled cells in them, and capture photographic images. A small anterior patch of the larval epithelium that included the labeled ASO cells was located with white light and a 40× objective. This area was then exposed to a narrow light beam (20 ± 2 μm) from a mercury source passed through a Zeiss fluorescein filter cube (excitation range 450–490 nm) for 1, 5, 10, 15, or 20 min. After treatment, the larvae were carefully cut free from the agarose, whereupon they resumed swimming in FSW.

Induction of metamorphosis in treated and untreated larvae. After being freed from the agarose, treated larvae were placed in petri dishes containing 5 ml of FSW that had been exposed to the coral *Porites compressa* for 12 h and filtered prior to use. Seawater conditioned with this coral prey of adult *P. sibogae* typically induces settlement and metamorphosis in 90%–100% of competent larvae in 24 h or less (*e.g.*, Pires *et al.*, 1997). The percentage of larvae that had metamorphosed was determined after 24 h; metamorphosis in *P. sibogae* is dramatic and easy to discern (Hadfield, 1978). Each treatment was replicated three times with 15 larvae per replicate. Controls to determine that larvae were metamorphically competent and that they did not metamorphose spontaneously were used in larger numbers (25–50), because it was not necessary to embed these larvae in agarose to make these determinations. The major methods used in the experiments described above are outlined in Figure 1; obvious non-imbedded controls (see below) are not included.

To test whether larvae could be induced to metamorphose with potassium or cesium after the photoinactivation treatment described above, in a separate experiment, treated larvae were released from agarose, allowed to swim in FSW for an hour or more, and then placed in FSW containing either 20 m*M* added potassium or 20 m*M* cesium. Because the maximal metamorphic response to K^+ typically takes about 48 h (Pechenik *et al.*, 1995), these experiments were run for 2 days, and the number that had metamorphosed was determined at 24 and 48 h. Larvae were exposed to cesium seawater for 20 min, returned to FSW, and examined to determine the percentage that metamorphosed after 24 h. Each of three replicates for each variable contained 15 larvae.

Controls. Untreated sibling larvae from the stock culture were employed to determine that larvae were competent to metamorphose when exposed to the coral-conditioned FSW and that larvae did not metamorphose spontaneously without exposure to coral water or other inducers. Larvae were neither decalcified nor embedded in agarose for these controls. Only batches of larvae that yielded 90%–100% metamorphosis within 24 h of exposure to coral water were used in the experiments. To test that the treatment effects (*i.e.*, DASPEI staining plus irradiation of the stained ASO) were those responsible for observed results: (1) larvae were stained with DASPEI but not exposed to 450–490 nm light; (2) unstained larvae were exposed to irradiation focused on the ASO for 20 min; and (3) stained larvae were exposed to irradiation focused on regions of the larvae body other than the ASO. In all of these controls, the larvae were subjected to the same treatments as the experimental larvae, including having their larval shells decalcified and being embedded in low-melting-point agarose.

Acridine orange staining to detect cell death. Untreated control larvae (also decalcified and embedded in agarose) and larvae that had been stained with DASPEI and exposed to radiation focused on the ASO were placed in a seawater solution of acridine orange (2.7×10^{-6} *M*) for 20 min. After exposure to acridine orange, the larvae were examined and photographed on a Zeiss fluorescence microscope

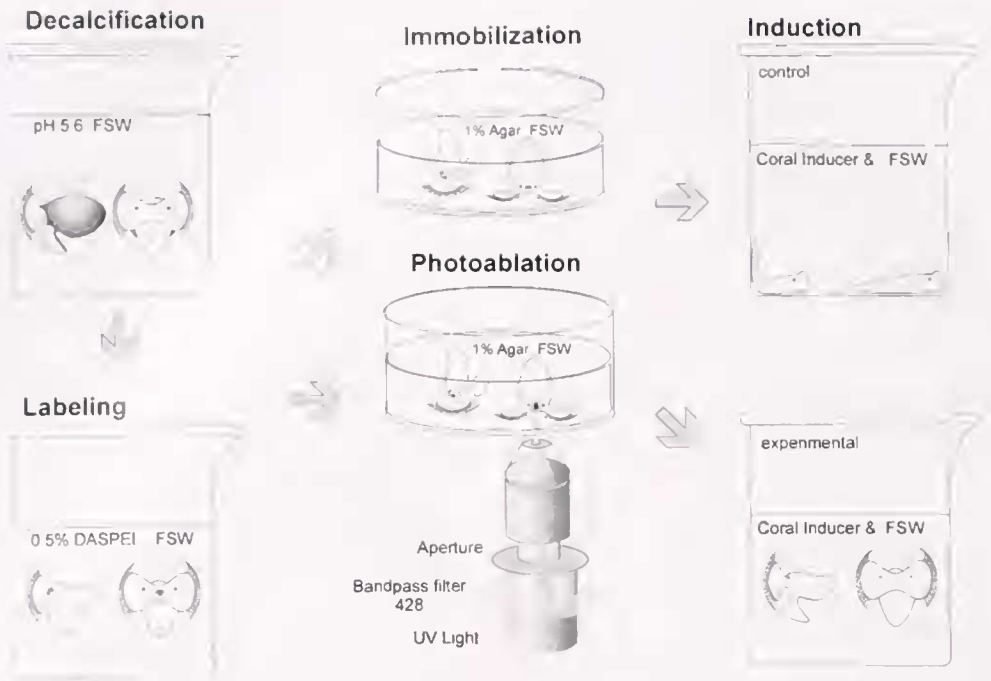

Figure 1. Diagram representing the methods used in photoablation of cells in the apical sensory organ of veliger larvae of *Phestilla sibogae*. Larval shells are decalcified without harm to the larvae, and then the larvae are exposed to DASPEI. Once immobilized in low-melting-point agarose, the larvae are put on the stage of an inverted fluorescence microscope and a 20-μm band of 450–490 nm light is focused on stained cells in the apical sensory organ. The larvae are then freed from agarose and, when swimming, exposed to the metamorphosis-inducing coral extract. Control treatments of unstained larvae are not illustrated.

equipped with filters that include the 436-nm excitation peak for acridine orange. The goal of this experiment was to determine if ASO cells in larvae that had been treated would take up acridine orange while those of untreated larvae would not. Uptake of acridine orange only by treated cells would provide some evidence for cell death, thus photoablation, of those cells.

Data were analyzed with Sigma Plot software, version 5.0 (Jandel Scientific Software Inc.). In instances where there were questions about differences between experimental response variables and controls, the proportional data were subjected to arc-sine transformation and the means compared with 2-sample t tests.

Results

Many but not all larvae that were exposed to DASPEI for 30 min and allowed to wash for one h in FSW displayed specific anterior staining in the ASO (Fig. 2). The locations and numbers (typically 5–6) of stained cells are consistent with their identity as components of the ASO. Unstained larvae displayed no autofluorescence in the ASO. The large prototrochal cells of the vela stained lightly with DASPEI, and various structures in the visceral hump, exposed by decalcification of the larval shell, also absorbed DASPEI, but these regions were easily excluded from the irradiated area. DASPEI was retained by larvae in FSW for periods in excess of one week, even through metamorphosis.

Larvae that had been stained with DASPEI, embedded in low-melting-point agarose, and irradiated with a narrow band of 450–490 nm light focused on the ASO survived well (>90%). When released from agarose and exposed to coral seawater, the typical metamorphic inducer, these larvae metamorphosed in percentages inversely proportional to the duration of irradiation (Fig. 3). The excitatory illumination resulted in photobleaching of the ASO cells; that is, they became colorless. DASPEI-stained cells in non-illuminated parts of the larvae continued to show brilliant fluorescence (Fig. 5A). Very few larvae that had been exposed for as long as 20 min were capable of metamorphosing in response to natural inducer. Excitatory illumination alone did not reduce the ability of larvae to metamorphose in response to coral inducer (Fig. 3, differences in responses in the control labeled P20-CI were not significantly different [$P > .190$] from those in the control labeled CI). DASPEI-stained larvae exposed to the irradiation beam focused on the larval foot instead of on the ASO retained full competence to metamorphose when exposed to the coral inducer (Fig. 4). There were no DASPEI-stained cells in the region

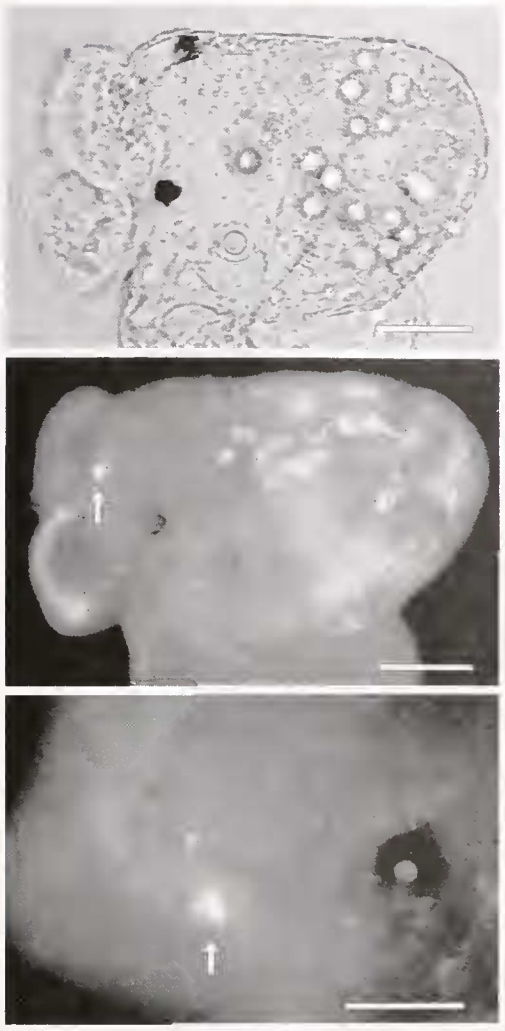

Figure 2. Veliger larvae of *Phestilla sibogae* stained with DASPEI. (top) Bright-field image, (middle) Same specimen photographed with fluorescent light. (bottom) Enlargement of anterior region of the same specimen showing DASPEI staining in 5–6 cells of the apical sensory organ. Scale bars, top and middle = 50 μm, bottom = 25 μm.

irradiated. We conclude that exposure of the stained ASO to intense excitatory irradiation for 20 min resulted in photoablation of the ASO cells, and that subsequent loss of the ability of these larvae to respond to the metamorphic inducer contained in coral seawater is evidence that the irradiated cells were the site of receptors for the inducer. These larvae were otherwise unharmed. They continued to swim normally in FSW, and they had not lost the capacity to metamorphose in response to artificial inducers (see below). The yellow DASPEI fluorescence disappeared from the ASO following excitatory illumination, although it persisted in other regions of the larval body.

When larvae whose ASO cells had been stained with DASPEI and exposed for 20 min to fluorescent irradiation and thus photobleached were placed in a seawater solution of acridine orange, the ASO cells uniquely absorbed the dye and emitted a green fluorescence (Fig. 5). Comparable staining in other cells and organisms has been found specific to induced cell death (Delic *et al.*, 1991; Abrams *et al.*, 1993). ASO cells of untreated larvae did not take up acridine orange. These observations support the conclusion that DASPEI staining followed by fluorescent irradiation of the ASO cells led to their ablation.

When larvae that had undergone the photoablation treatment described above were exposed to seawater containing 20 m*M* cesium ion for 20 min and then transferred to FSW, they underwent normal metamorphosis in large numbers within 24 h (Fig. 6). Similarly treated larvae exposed to seawater containing 20 m*M* excess potassium also metamorphosed in numbers much greater than controls, reaching more than 50% after 48 h (Fig. 6). The latency of the metamorphic response of larvae of *Phestilla sibogae* to potassium ion has been reported previously (Pechenik *et al.*, 1995). Control exposures of treated larvae to coral inducer demonstrated that they were, as in the experiments described above, unresponsive. Low percentages of larvae that metamorphosed after photoablation without inducer (Fig. 6, C-2) or with inducer (Fig. 6, CI in Photoablation bracket) were not significantly different from those in untreated larvae (Fig. 6, CI) (*t* tests, $P \gg 0.05$). We conclude that the site of action of K^+ and Cs^+ in inducing metamorphosis is not on the primary receptor cells, which had been destroyed in the experimental treatment. However, the possibility of multiple sites of metamorphic stimulation by these cations is not eliminated.

Discussion

The data presented here provide compelling evidence that cells in the apical sensory organ bear the receptors for the dissolved molecular inducer of settlement and metamorphosis in larvae of the gastropod *Phestilla sibogae*. Evidence presented by Wodicka and Morse (1991) and Baxter and Morse (1992) demonstrated that receptors for a related receptor pathway are found on cilia harvested from competent larvae of another gastropod, *Haliotis rufescens*. Yool (1985) had earlier demonstrated that these larvae have an apical ciliary structure that is presumably homologous to the ASO of *P. sibogae*. It remains to be demonstrated if the key pathway, that for the settlement/metamorphic inducer (for which GABA is an agonist), is also on the same cilia in larvae of *H. rufescens*. Apical sensory organs have been demonstrated in all opisthobranch larvae that have been examined appropriately (Bonar, 1978; Chia and Koss, 1984; Kempf *et al.*, 1997; Marois and Carew, 1997), as well as in two prosobranch species (Uthe, 1995; Leise, 1996). While it is logical to assume that ASOs in other gastropods have a role in metamorphic induction, experimental evidence for this sensory function has not yet been provided.

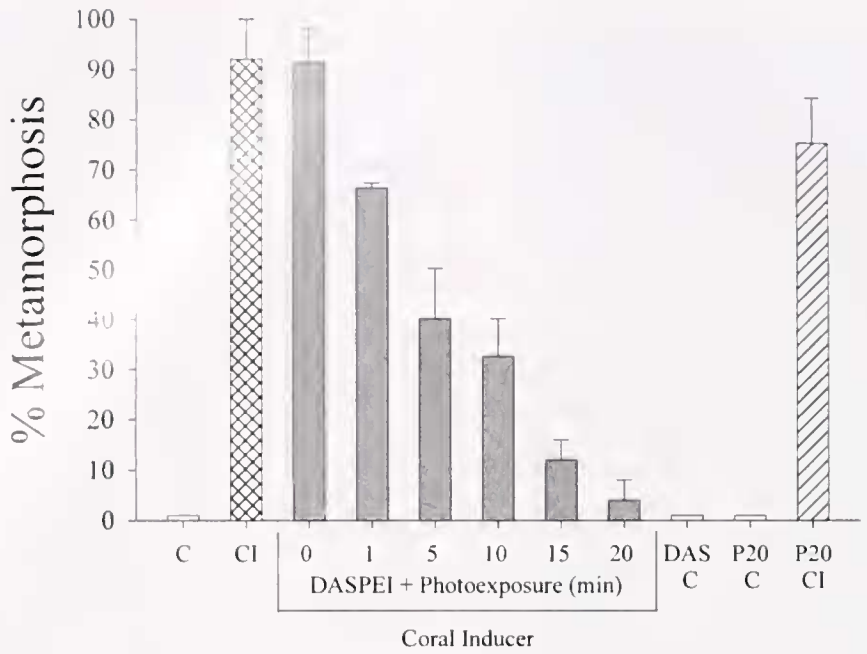

Figure 3. Percent metamorphosis in larvae of *Phestilla sibogae* that have been stained with DASPEI, exposed to fluorescent illumination focused on the stained cells in the apical sensory organ, and exposed to coral metamorphic inducer; bars = mean and SD. Experimental and control larvae embedded in agarose (n = 3 replicates, 15 larvae/replicate): DASPEI + Photoexposure, DASPEI-stained larvae exposed to a beam of fluorescent light focused on stained cells of the apical sensory organ for 0–20 min and then placed in coral inducer for 24 h; DAS-C, DASPEI-stained larvae that were not photo-treated nor exposed to coral inducer; P20-C, unstained larvae exposed to fluorescent light for 20 min and not placed in coral inducer; P20-CI, unstained larvae exposed to fluorescent light for 20 min and then placed in coral inducer. CI, untreated larvae exposed to coral inducer for 24 h. Controls with larvae *not* stained nor embedded in agarose (n = 3 replicates, 25 larvae/replicate): C, control to determine that the batch of larvae did not show high levels of spontaneous metamorphosis; CI, control to determine that the batch of larvae metamorphosed normally upon exposure to the coral inducer without other treatment. P20-CI was not significantly different from CI (t test, $P > 0.190$).

The homology of key elements of the apical sensory organ of gastropod veligers, and presumably those of other molluscan larvae (Raven, 1966, pp. 143–145), with the apical-tuft/apical-organ complex of the trochophore larvae of polychaetous annelids appears to be sound. The cell lineage for the ASO of larvae of the gastropod *Crepidula fornicata*, described by Conklin (1897), appears to be identical to that for the apical organ of polychaete trochophores; in both larval types, the apical organs arise from descendants of the first-quartet micromeres $1a^1$–$1d^1$ (summarized by Kume and Dan, 1968). Cells bearing apical-tuft cilia appear to arise from the same micromere quartet in polyclad flatworms (Selenka, 1881) and nemerteans (Hörstadius, 1937) that produce pelagic larvae. There thus appears to be great antiquity in the apical sensory organs of larvae of spiralian metazoans, although functional similarities remain to be demonstrated in most cases.

The chemical cues that trigger metamorphosis in larvae of *Phestilla sibogae*, at least some other gastropods, and a number of other invertebrate types are dissolved in seawater (nudibranchs: Thompson, 1958; Hadfield and Scheuer, 1985; Bahamondes-Rojas and Dherbonez, 1990; Lambert and Todd, 1994; prosobranchs: Scheltema, 1961; McGee and Targett, 1989; Boettcher and Targett, 1998; bivalves: Zimmer-Faust and Tamburri, 1994; sipunculans: Rice, 1986; echinoids: Burke, 1984; Pearce and Scheibling, 1990a, 1990b; ascidians: Young and Braithwaite, 1980; barnacles: Rittschof, 1985; crabs: Welch *et al.*, 1997). In contrast, many other larvae detect cues that are absorbed to, or are part of, the chemical structures of more-or-less specific surfaces. Surface-bound cues to settlement for benthic invertebrates include the algal-surface ligands to which larvae of *Haliotis rufescens* respond (Morse and Morse, 1984), barnacle-associated substances that serve as a settlement cue for the barnacle-eating nudibranch *Onchidoris fusca* (Arkett *et al.*, 1989), and bacterial-biofilm substances that stimulate settlement and metamorphosis in competent nectochaete larvae of the polychaete *Hydroides elegans* (Carpizo-Ituarte and Hadfield, 1998) and many other invertebrates. Amazingly few detailed descriptions of larval behavior prior to settlement have been published. However, at least some polychaete trochophores (Nott, 1973; unpub-

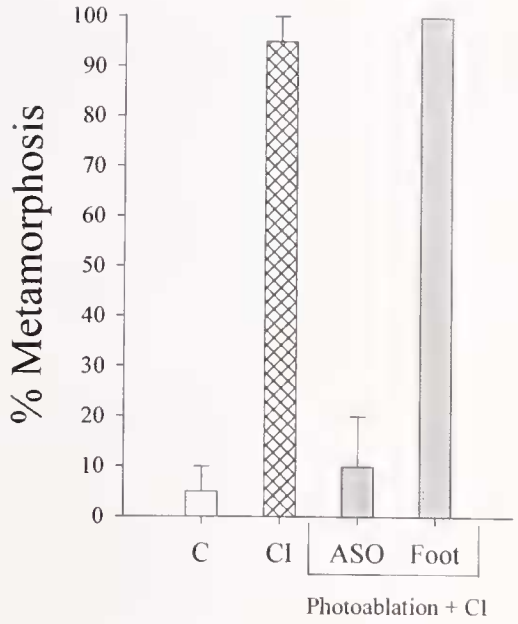

Figure 4. Percent metamorphosis in larvae of *Phestilla sibogae* that were stained with DASPEI, embedded in low-melting-point agarose, exposed for 20 min to fluorescent irradiation aimed either at the apical sensory organ (ASO) or the foot, freed from the agarose, and then placed in coral inducer for 24 h (n = 3 replicates, 15 larvae/replicate); bars = mean and SD. ASO, fluorescent light focused on the ASO; Foot, fluorescent light focused on the foot. Not embedded in agarose were larvae used in C, untreated controls; CI, untreated larvae exposed to coral inducer (n = 3 replicates, 25 larvae/replicate).

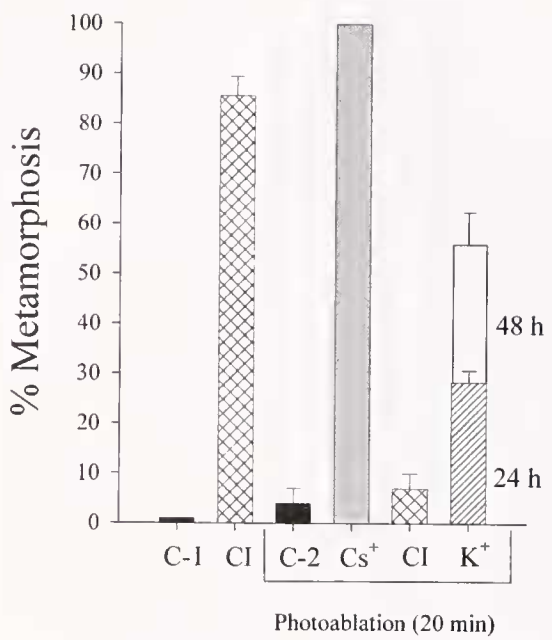

Figure 6. Percent metamorphosis in larvae of *Phestilla sibogae* that had undergone photoablation of apical-sensory-organ (ASO) cells and then exposure to the ionic inducers potassium or cesium; bars = mean and SD. Percent metamorphosis was determined after 24 h in all cases, plus at 48 h for potassium-treated larvae. Experimental treatments (larvae embedded in low-melting-point agarose for light treatment; n = 3 replicates, 15 larvae/replicate) in Photoablation (20 min) bracket, all stained with DASPEI and subjected to 20-min excitatory illumination focused on the ASO: C-2, larvae not placed in coral inducer; CI, larvae exposed to coral inducer; Cs^+, larvae exposed to 20 m*M* cesium chloride in seawater for 20 min; K^+, larvae continuously exposed to seawater augmented with 20 m*M* excess potassium chloride. Controls (not embedded in agarose): C-1, untreated larvae not exposed to coral inducer; CI, untreated larvae exposed to coral inducer for 24 h (n = 3 replicates, 25 larvae/replicate). Neither C-2 nor CI with photoablation treatment were significantly different from the untreated-larvae control C-1 (t tests, $P \gg 0.05$).

lished personal observations on larvae of *Hydroides elegans*), larvae of a chiton (Barnes and Gonor, 1973), and veligers of the abalone *Haliotis rufescens* (Wodicka and Morse, 1991) apparently detect absorbed settlement cues by brushing the surfaces of substrata with their apical ciliary tufts during a period before settlement and attachment. We presume that during what has been interpreted as pre-attachment "searching behavior," the larvae apply chemically sensitive cilia to potential settlement sites in a manner that will bring together stimulatory ligands and their specific receptors and initiate neurological signaling for settlement and metamorphosis. Because cilia protrude very little, if at all, from the sensory cells in the apical sensory organ of veligers of *P. sibogae*, they would be of little use in contact chemoreception; additionally, the cilia of these cells are structurally similar to stereocilia—as contrasted with motile cilia—such as those found in other sensory organs (Bonar, 1978). The same is true of the apical sensory cells of veligers of the prosobranch *Littorina littorea* (Uthe, 1995).

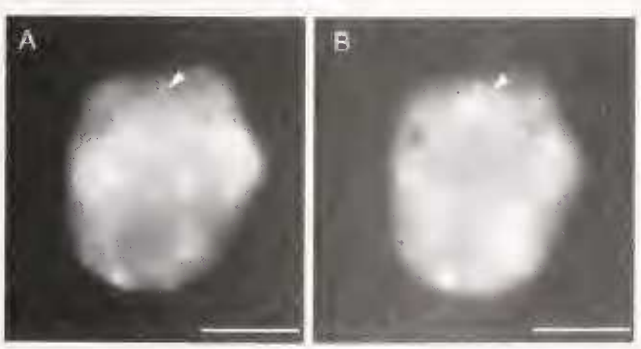

Figure 5. (A) Veliger larva of *Phestilla sibogae* that was stained with DASPEI, embedded in low-melting-point agarose, and subjected to excitatory irradiation focused on the apical sensory organ (ASO) for 20 min. The ASO cells are bleached (compare to Fig. 2B, C), although the DASPEI stain remains in other parts of the larval body. (B) The same larva after 20-min immersion in acridine orange in filtered seawater. The ASO cells emit a pale green fluorescence. Other bright areas, some not visible in both photos, retain DASPEI stain. Scale bars = 100 μm.

Kempf *et al.* (1997) provided detailed ultrastructural and immunocytochemical evidence for the presence of at least three cell types in the apical sensory organs of nudibranch larvae. Three sensory serotonergic neurons are intricately associated with the innervation of the velum, leading the

authors to conjecture that these elements of the ASO are mechanosensory and serve as a compensatory system of velar control to "modulate the position of the velar lobes in response to deformations of the pretrochal surface caused by changes in velar orientation." It appears most likely that the cells responsible for chemosensory detection of the metamorphic cue in larvae of *Phestilla sibogae* are the so-called flask-shaped cells (Bonar, 1978; = ampullary cells of Chia and Koss, 1984). These fit in number (five or six in veligers of *P. sibogae* according to Bonar's [1978] ultrastructural study) and location with those that were stained by DASPEI in the current study (Fig. 2), and cells with very similar structure have been shown to be chemosensory in a cephalopod (Lucero *et al.*, 1992).

Acridine orange has long been employed as a vital stain for dying eukaryotic cells (reviewed by Darzynkiewicz and Traganos, 1998). When complexed with DNA, acridine orange fluoresces in the green part of the spectrum (525 nm); as a weak stain for RNA, its emission is reddish (~650 nm) (Haugland, 1996). Although some authors (*e.g.*, Abrams *et al.*, 1993) maintain that acridine orange staining differentiates apoptotic cells (*i.e.*, those dying from "programmed cell death") from necrotic cells, others report the opposite. Darzynkiewicz and Traganos (1998, p. 55) note that both late apoptotic and necrotic cells show green fluorescence at low (*i.e.*, $\sim 10^{-6}$ *M*) concentrations of acridine orange. Experiments conducted by Delic *et al.* (1991, p. 147) demonstrated that acridine orange "intercalates into nuclear DNA following the discharge of lysosomal enzymes *via* a targeted photodynamic reaction triggered by high levels of light intensity." Thus the specific uptake of acridine orange by the larval ASO cells after the photoinactivating treatment employed here appears to support the conclusion that DASPEI staining followed by excitatory irradiation has killed the cells. The applied concentration was in the micromolar range, and cells treated by a presumed photodynamic interaction of UV light with a fluorescent stain brought about the cell death.

The mechanism by which potassium and cesium ions induce metamorphosis in various invertebrate larvae has been a subject of considerable conjecture (*e.g.*, Yool *et al.*, 1986; Todd *et al.*, 1991; Herrmann, 1995; Pechenik *et al.*, 1995; Woollacott and Hadfield, 1996; Carpizo-Ituarte and Hadfield, 1998). A leading hypothesis has centered on the likelihood that elevated external potassium depolarizes excitable sensory cells, presumably those bearing the receptors for external chemical metamorphic inducers, causing these cells to generate electrical spikes that are transmitted through the central nervous system to bring about morphogenesis (*e.g.*, Baloun and Morse, 1984). Cesium, acting as a potent blocker of potassium channels, has been thought to have a similar action (*e.g.*, Carpizo-Ituarte and Hadfield, 1998). However, nearly all those who have carried out such experiments have acknowledged that entire larvae are bathed in the seawater with elevated potassium or cesium ions and that the ions could be acting downstream from the primary chemosensory cells (Todd *et al.*, 1991). We have presented here data consistent with the latter hypothesis, although exactly where downstream awaits clarification. The possibility that K^+ and Cs^+ act on external receptors as well as downstream sites in intact larvae is, of course, not disproven by these experiments. There are undoubtedly numerous synapses in the central nervous system between the ASO and the responding tissues where potassium and cesium could act.

Because it focused on a single gastropod species, the current research provides no support for homology of ASO cells among gastropods or across phyla. However, it does point to profitable future research on this subject. (1) Electron-microscopic study of the ASOs of larvae of *P. sibogae* after the photoablation treatment could reveal exactly which cells were affected, and thus confirm that the flask-shaped cells with nonmotile cilia are the critical receptor cells in metamorphic signaling. (2) If the experiments described here were performed with larvae of other species, across the Mollusca and other spiralian phyla, and similar results obtained, the data would be further evidence for homology. The new data presented here, considered together with experimental data on the molecular attributes of metamorphic-cue receptors in larvae of the abalone (Baxter and Morse, 1987) and the polychaete *Hydroides elegans* (Carpizo-Ituarte and Hadfield, 1998; Holm *et al.*, 1998), provide a framework for investigating the molecular nature of the receptor molecules used by these larvae in settlement-cue perception and the homology of these receptor molecules across phyla. Data on the abalone, *H. elegans*, and *P. sibogae* (Hadfield, unpubl. obs.) strongly suggest that these receptor molecules are *not* members of the G-protein-transduced, 7-transmembrane-domain peptides typical of much chemical perception in animals. Once the specific location of the actual receptor molecules is demonstrated across phyla, and their molecular sequences are known, molecular probes (*e.g.*, *in situ* hybridization) could be employed to provide additional evidence for homology and phylogeny. Similar experimental data obtained from other phyla, especially the noncoelomate spiralians, would greatly expand our understanding of the evolution of the ASO complex and, perhaps, evolutionary relationships among the spiralians.

Acknowledgments

This research was supported by ONR grants N00014-94-1-0524 and N00014-95-1-1015. Many members of our research group at the University of Hawaii's Kewalo Marine Laboratory contributed significantly to maintaining research animals, culturing larvae, and assisting in the research at important moments; we thank them all. We thank Carole Hickman (University of California, Berkeley) for her con-

structive criticisms and suggestions for improving the manuscript, Richard Strathmann (University of Washington) for assisting the authors in recalling older references on larval settlement behavior, and two anonymous reviewers for suggestions that clarified significant parts of this paper.

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